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16. ABSTRACT
There are demonstrable associations in experimental animals between DNA damage in somatic cells and the development of cancer, and between DNA damage in germ cells and the incidence of genetic disease in offspring. Thus, there is substantial evidence of the need to detect and to quantitate chemically-induced genetic damage in humans in order to assess the potential for cancer and genetic disease. Current methods for estimating human risk of cancer have been based on knowledge of human exposure and epidemiological data. An alternative approach would base such estimates of risk on knowledge of exposure and of damage to the DNA of human cells and tissues. The principal difficulty in performing such assessments lies in the uncertainty of translating information from tests for genetic damage in human cells and tissues into reliable estimates of risk for cancer or genetic disease. This poster concerns: (1) the development of mammalian cell methods that may be used ultimately to evaluate genotoxic effects in humans and (2) our initial attempts to implement a "parallelogram" approach whereby such methods can be used, collectively, to relate quantitative information on genetic damage to quantitative estimates of risk for cancer or genetic disease.

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EVALUATION OF GENOTOXIC EFFECTS IN HUMAN POPULATIONS

PB84-128982

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1. INTRODUCTION

1.1. Need to Detect Chemically Induced Genetic Damage in Humans

There are demonstrable associations in experimental animals between DNA damage in somatic cells and the development of cancer, and between DNA damage in germ cells and the incidence of genetic disease in offspring. Thus, there is substantial evidence of the need to detect and to quantitate chemically induced genetic damage in humans in order to assess the potential for cancer and genetic disease. Current methods for estimating human risk of cancer have been based on knowledge of human exposure and epidemiological data. An alternative approach would base such estimates of risk on knowledge of exposure and of damage to the DNA of human cells and tissues. The principal difficulty in performing such assessments lies in the uncertainty of translating information from tests for genetic damage in human cells and tissues into reliable estimates of risk for cancer or genetic disease.

This paper concerns: (1) the development of mammalian cell methods that may be used ultimately to evaluate genotoxic effects in humans and (2) our initial attempts to implement an approach whereby such methods can be used, collectively, to relate quantitative information on genetic damage to quantitative estimates of risk for cancer or genetic disease. Such an approach is not new, having been advocated previously by Sobels[1,2], Malling[3], and Lyon[4].

The "parallelogram method" is an approach involving somatic- and germ-cell monitoring techniques whereby quantitative dose-response relationships are

developed among component test systems. With the parallelogram method, illustrated in Figure 1, estimates of chemical (external) exposure and cellular and molecular (internal) dose provide the quantitative basis for extrapolating data from whole animal systems to human populations. The use of animal and human cells in vitro enables direct comparisons of induced effects under rather carefully controlled dosages. Such comparisons have conventionally been based on measurements of total dose to the DNA or common molecular products in the DNA determined after exposure of the organism to the agent in question.

The relative mutagenic effects of individual agents may be subject to further quantitation if the kinds of DNA adducts formed and their distribution within the molecule after exposure can be characterized in the various test systems and tissues. A vast literature already exists concerning the products of chemical interactions with DNA and the initiation of carcinogenesis. Singer[5], in classifying the reaction products formed by the alkylation of nucleic acids and polynucleotides, found the correlation with carcinogenicity to be good for certain alkylation products. Singer also reported that the relative amount of specific alkylated derivatives of DNA is similar, even though total alkylation may vary a thousandfold for DNA. This observation holds for analyses made in the test tube, in intact cells, or in the whole animal[5]. In the final analysis, the reaction products formed by the interaction of chemicals with DNA are of consequence for assessment purposes only if they relate directly to genetically significant targets.

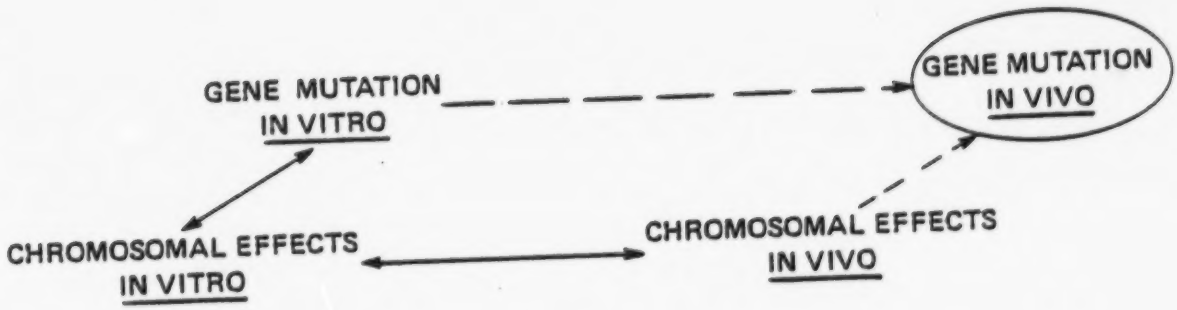


Figure 1a. Parallelogram for extrapolation to gene mutation in vivo.

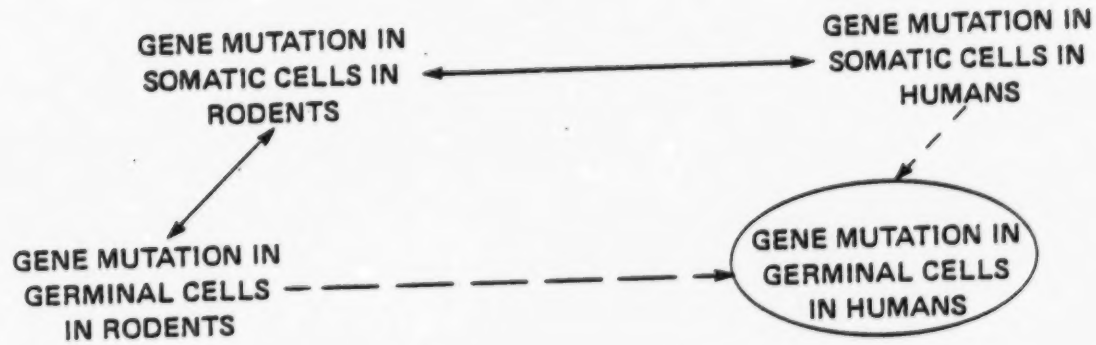


Figure 1b. Parallelogram for extrapolation to gene mutation in germinal cells in humans.

1.2 Biological and Dosimetric Methods Available

Under certain conditions, genotoxic damage can be measured directly in acutely exposed humans. However, if the genetic damage is repaired, little or no evidence may exist that can be related to heritable mutations or tumors in the exposed population. Studies using low chronic exposures are also limited, because they may not produce a measurable genotoxic endpoint or evidence of an increased disease frequency. Thus, within the limits of current methodology, some combination of animal and short-term genetic tests must be used to supplement any available human exposure data.

The relationships presented in Figure 1 and expanded in Figure 2 illustrate the parallelogram method of predicting genotoxic effects in humans. As illustrated, external exposure (e.g., ambient air) or internal exposure (e.g., intraperitoneal injection) can be functionally related to mutation or tumor incidence in whole animal or short-term test systems. Data from these test systems can, in turn, be used to estimate effects in humans under similar exposure conditions. Estimates are made in a stepwise fashion and are corrected by factors that account for differences in test system sensitivity, size differences between species, etc. If uncertainties in this approach can be held to a minimum, one or more test systems then can be used to assess possible carcinogenicity or heritable mutation in humans.

As indicated by the arrows in Figure 2, one species system (e.g., mouse) can be used to relate external exposure to internal exposure (blood level) and internal exposure to the level of DNA alkylation in germ tissue. However, this

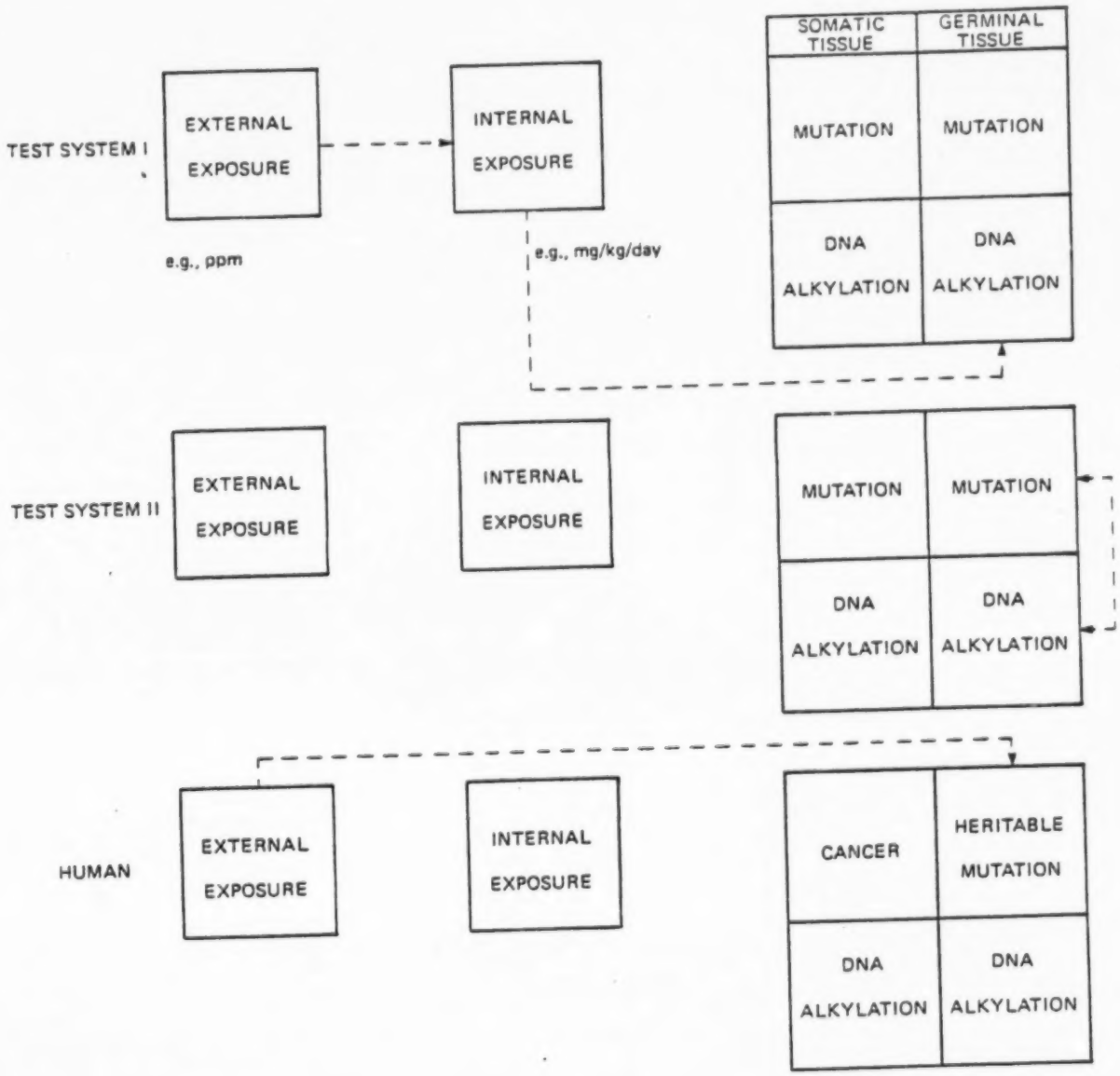


Figure 2. Expanded concept of parallelogram method considering external and internal exposure.

species may not be the best one for linking the level of DNA alkylation to mutation in germ tissue. This may be effectively achieved with a different species (e.g., Drosophila), which allows DNA alkylation to be functionally related to mutation in germ tissue. The surrogate species is useful, providing that DNA alkylation and gene mutation are parallel in the two species over the dose range considered. Such comparative dosimetry forms the quantitative basis of the assessment process referred to as the parallelogram method[6-11].

1.3 Chemicals Under Investigation

Several chemical agents are under investigation in this laboratory for which parallel studies in animal and human assay systems in vitro and in vivo are feasible. These include the cancer chemotherapeutic agent, cyclophosphamide, and the industrial intermediate, ethyl carbamate (urethane). Each of these compounds presents a useful model for the purposes of our investigations. The former agent is of substantial clinical relevance and may be useful in further development of the parallelogram approach. The latter agent is of perhaps greater theoretical significance in terms of our lack of understanding of its metabolism, especially in short-term tests, and with regard to the difficulties in extrapolating data from such tests to experimental animals and humans.

Cyclophosphamide is an alkylating nitrogen mustard: its reactive moiety is the bis(2-chloroethyl) group. The compound is widely used clinically for treatment of Hodgkin's Disease and in the treatment of lymphoma and leukemia, neuroblastoma, carcinoma of the breast, and a variety of other malignant

diseases. The agent is also used as an immunosuppressant in several types of nonmalignant diseases, including rheumatoid arthritis, nephrotic syndrome in children, and chronic hepatitis. Hence, there are substantial opportunities to obtain human exposure and effects data with this compound.

Ethyl carbamate (EC) is considered a "problem carcinogen" because of erratic or generally negative genetic test results[12]. EC was one of the earliest known chemical carcinogens[13], and it has continued to be a public health concern because of its former use in medicine and current widespread use in industry[14]. Of several "model rodent carcinogens" recently studied, EC alone also proved to be carcinogenic for Old and New World species of monkey[15]. Despite 40 yr of various toxicological studies with EC, it still is not understood in terms of mechanistic pathways leading to pathology. Covalent binding to DNA is known to occur[16]; however, EC has proven relatively inactive in most short-term tests for carcinogens[12,17]. Therefore, EC provides a useful, albeit difficult, "model carcinogen" for comparative studies in genetic test systems.

2. METHODOLOGY

2.1. Introduction to Component Tests

The following section describes the component test methods being employed in this laboratory to measure chromosomal effects and gene mutation, and to provide biological evidence of exposure (urine screening). Results obtained from such biological measurements will be integrated using appropriate

dosimetric techniques. The specific test methods to be described are:

- Methods for measuring chromosomal effects
  - Sister chromatid exchange, *in vitro* and *in vivo*
  - Aberrations, *in vitro* and *in vivo*
- Methods for measuring (putative) gene mutation
  - HGPRT, fibroblasts, *in vitro*
  - HGPRT, lymphocytes, *in vitro* and *in vivo*
  - TK, lymphoma cells, *in vitro*
- Methods for measuring exposure
  - Urine screening with *Salmonella typhimurium*
- Dosimetry methods
  - Total DNA adducts

2.2. Description of Test Methods

2.2.1. Methods for Measuring Chromosomal Damage

Major areas of cytogenetic investigation include human monitoring, and *in vitro* and *in vivo* mammalian cell studies. These areas are discussed below.

2.2.1a. Human Monitoring. Two major cytogenetic endpoints are evaluated in human monitoring studies--chromosome aberrations and sister chromatid exchange (SCE) induction. Peripheral lymphocytes are obtained from individuals exposed to a chemical agent and cultured in an appropriate growth medium containing phytohemagglutinin (PHA) to stimulate cell cycling. Standard

procedures[11,18,19] for cell harvest, slide preparation, and aberration analysis are followed.

In the SCE studies, 5 x 10^6 lymphocytes are cultured in 10 ml of growth medium, to which 25 μmolar 5-bromodeoxyuridine (BrdU) is added after 24 hr[22] (Figure 3). Cells are harvested after 72 hr in culture (2 hr after a colchicine treatment to collect metaphases), by which time a high proportion of cells have cycled twice and become unequally substituted with BrdU in the sister chromatids. Cells are then stained with a BrdU-sensitive dye and examined for differentiated sister chromatids in which SCEs manifest themselves as reciprocal exchanges. First metaphase division cells can also be harvested at earlier times (e.g., 48-54 hr) and analyzed for chromosome aberrations. The alternative methods for aberration analysis (cells with or without BrdU substitution), the appropriate conditions under which aberration and SCE analyses are applied, and the individual strengths and weaknesses of the two endpoints have been reviewed[11,18-21]. For further details concerning the procedure described here, see Allen et al.[22].

2.2.1b. In Vitro Analyses. The in vitro studies routinely conducted involve the use of lymphocyte (human, rodent) or Chinese hamster V79 cells. Details concerning these analyses can be found in[22,23]. As shown in Figure 4, alternative activation sources and multiple genotoxic endpoints can be accommodated by the V79 system; the number of cells seeded varies with the activation system employed and the particular endpoint(s) evaluated. For activation, liver S9 enzymes or intact cells (e.g, hepatocytes) may be used. After test chemical treatment, cells are reseeded under separate growth

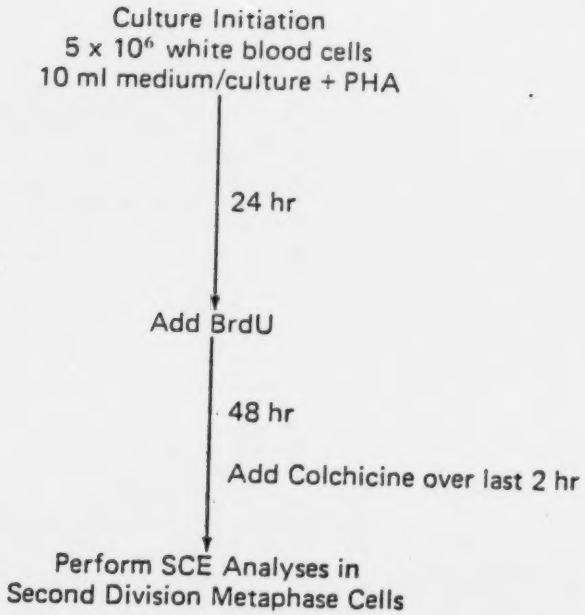


Figure 3. Protocol for culturing human lymphocytes for SCE analysis.

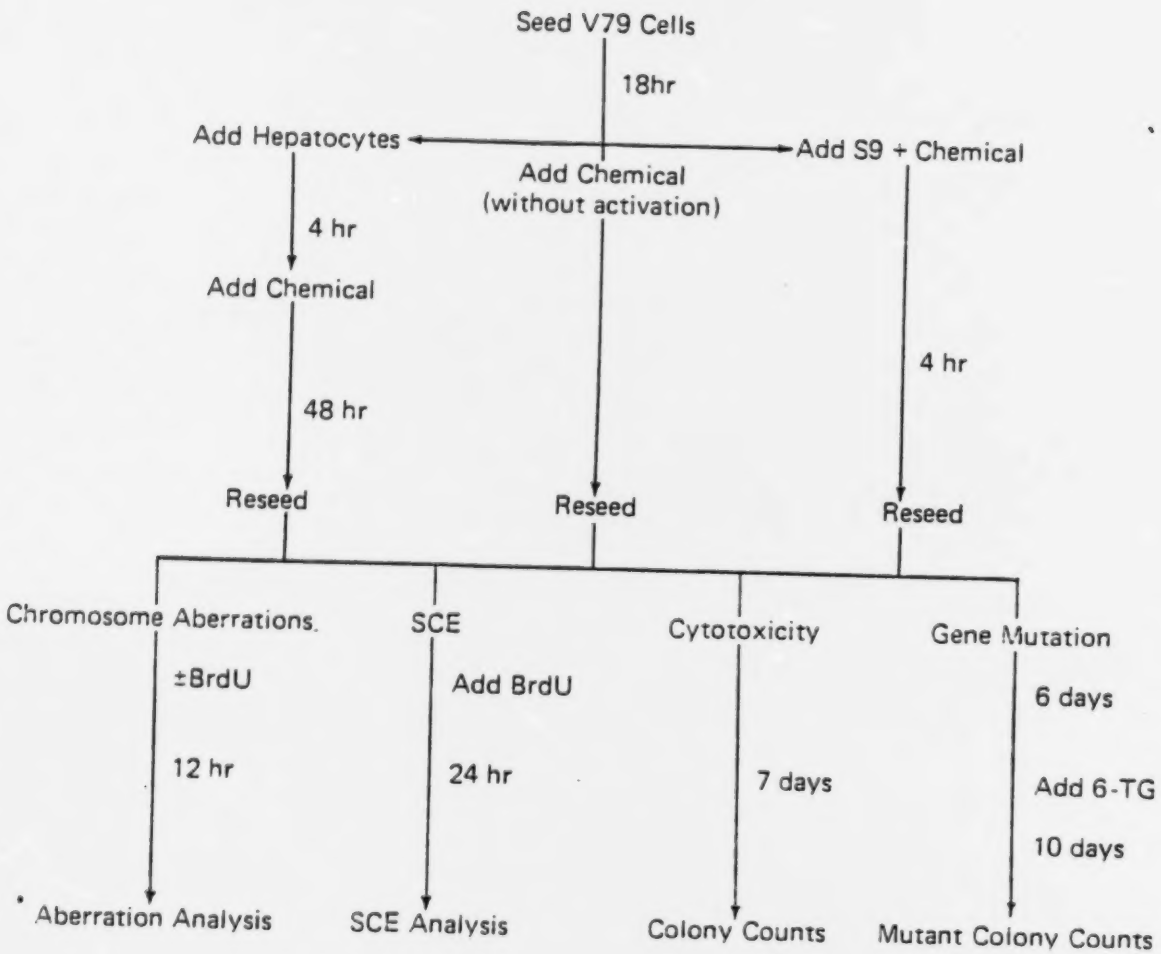


Figure 4. Protocol for <u>in vitro</u> analysis of chromosomal, gene, and cytotoxic effects from mutagens/carcinogens.

conditions for each endpoint being assayed, i.e., gene mutations (6-thioguanine resistance and/or ouabain resistance), cytotoxicity, chromosome aberrations, and SCE. With this approach, several endpoints are measured from the same exposure population, and the sensitivities of the different types of genetic damage can be readily compared.

2.2.1c. In Vivo Analyses. In vivo cytogenetic studies involve the use of mice, rats, or hamsters. SCE and chromosome aberration analyses are usually performed using bone marrow, regenerating liver, and/or spermatogonial cells. For details of these procedures, see Allen et al.[24], Latt et al.[11], and McFee et al.[25]. Aberrations are analyzed at the first metaphase division (approximately 12 hr in somatic cells, 26 hr in germ cells) after exposure to the test chemical. For SCE analyses (Figure 5), a BrdU tablet is implanted subcutaneously for DNA labeling and the test chemical is administered intraperitoneally, coincident with the start of either the first or second DNA synthesis period or just prior to BrdU treatment. Metaphases are collected by intraperitoneal injection of colchicine (approximately 2 hr before cell harvest); marrow cells are harvested and analyzed for SCE approximately 22 hr after the start of labeling. BrdU tablet methodology can also be used for studies of transplacental SCE induction in specific fetal rodent tissues[26].

2.2.2. Methods for Measuring Gene Mutation

Presumptive gene mutation is being measured in a variety of mammalian and human lymphocyte and fibroblast culture systems. Exposures are in vitro as well as in vivo.

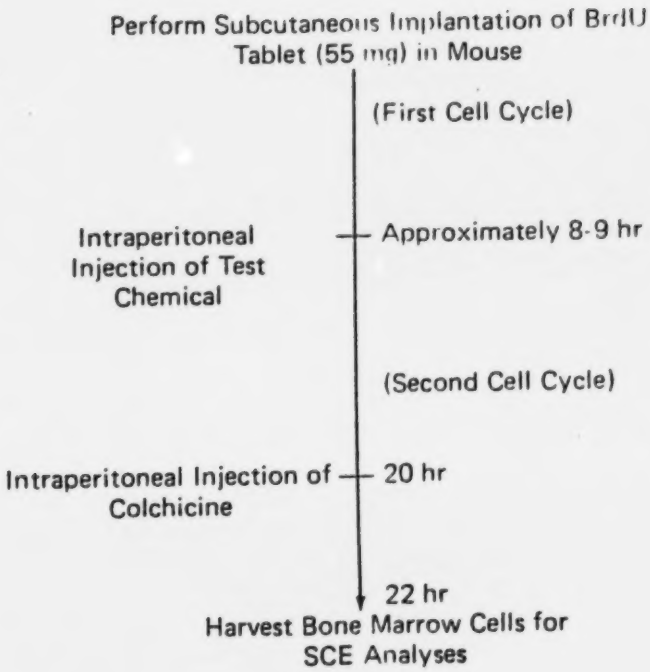

Perform Subcutaneous Implantation of BrdU Tablet (55 mg) in Mouse
(First Cell Cycle)
Intraperitoneal Injection of Test Chemical
Approximately 8-9 hr
(Second Cell Cycle)
Intraperitoneal Injection of Colchicine
20 hr
22 hr
Harvest Bone Marrow Cells for SCE Analyses

Figure 5. Protocol for in vivo analysis of SCE induction in rodent bone marrow cells.

2.2.2a. HGPRT, Fibroblasts--In Vitro. The method employed to detect gene mutation at the HGPRT locus in normal human fibroblasts was developed by Albertini and DeMars[27] using foreskin fibroblasts. In this method, the induction of 8-azaguanine (AG') or 6-thioguanine (TG') resistant cells is studied in the presence or absence of S9 activation.

Guanine, hypoxanthine, and 6-mercaptopurine are good substrates for HGPRTase, an enzyme that generally plays a salvage role in mammalian purine metabolism. HGPRTase plays a key role in the anabolism of 6-oxypurines and their analogs, converting azaguanine (AG) or thioguanine (TG) to nucleotides that inhibit the growth of normal fibroblasts. Because of their reduced ability to produce these nucleotides, HGPRT-deficient cells proliferate in the presence of AG or TG. It thus becomes possible to select these cells from a mutagenized cell population; the mutants resemble the naturally occurring Lesch-Nyhan (LN) cells.

The HGPRT locus is known to be on the X chromosome. Since males have a single X chromosome and females only one active X chromosome, mutations at the HGPRT locus should be readily recoverable as AG or TG resistance in cells of male or female origin.

Previous studies indicated that only S-phase cells were sensitive to mutagens, thereby implying that DNA replication was required for the induction of mutation[28-34]. Experiments have been conducted using synchronized human cells to detect mutagenic activity of several chemical agents, including cyclophosphamide. Unfortunately, experiments using synchronized cells receiving

only a single treatment of cyclophosphamide have failed to produce mutations. Studies using a different protocol, i.e., nonsynchronized cells receiving multiple treatments, have indicated that cyclophosphamide is mutagenic to human cells. The protocols used in these studies--single treatment in synchronized cells and multiple treatment in nonsychronized cells--are discussed below.

As illustrated in Figure 6, in tests involving single treatments, human fibroblasts are synchronized according to the method of Milo and DiPaolo[35]. With this method, 1.5×10^6 S-phase cells are exposed to the chemical test agent for 4 hr. The treated cells are then expressed in FCS-F10 medium (10% fetal calf serum in Ham's medium) for 7 days, with two passages. After 7 days, 10^4 cells are plated per 60-mm dish, and each dish receives TG at a final concentration of 7.5×10^{-5} moles. The selective medium is renewed once every week. In order to determine cloning efficiency, cells are plated (100 cells per 60-mm dish) and grown in FCS-F10 medium. Dishes used to estimate cell survival are stained at the end of the second week; dishes used to determine mutagenesis are stained at the end of the third week. Mutant colonies are then counted, and the mutation frequencies are determined based upon a Poisson distribution[27,36-39]. In order to test for promutagens, a treatment mixture consisting of a balanced salt solution and cofactors, 1 ml S9 fraction, mutagen dissolved in 0.1 ml dimethly sulfoxide, and 8.5 ml F10 medium is prepared. S-phase cells are exposed to the mixture incubated at 37°C for 4 hr. After a 7-day expression period, cells are plated and mutation frequencies are determined assuming a Poisson distribution.

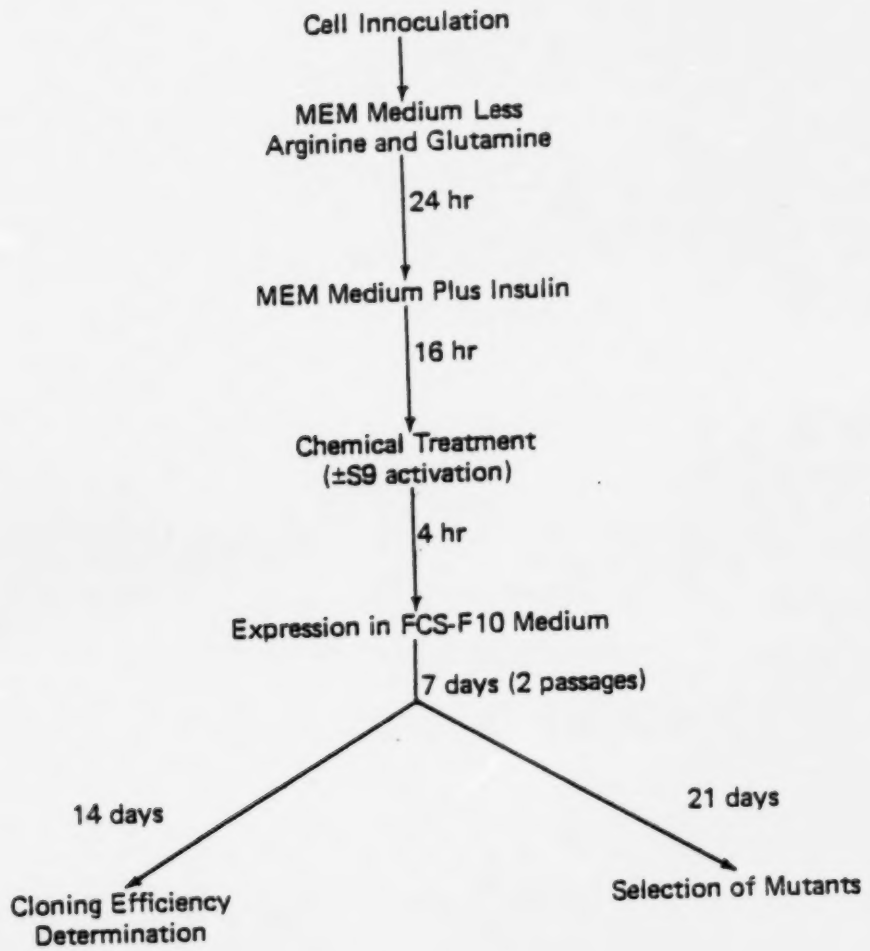


Figure 6. Induction of 6-thioguanine resistance studied in synchronized human fibroblasts in the presence or absence of S9 activation.

For tests involving multiple treatments (see Figure 7), 2.5×10^5 nonsynchronized cells per dish are exposed to the test agent for 4 hr daily for 3 to 4 days. Five or more dishes are used for each concentration. The treated cells are then expressed in FCS-F10 medium for 7 days, with two passages. Because of the toxic effect of S9, cell exposures to the treatment mixture and promutagen are limited to 4 hr each day with a reduced amount of S9, for 4 consecutive days. After each treatment, the S9 and mutagens are removed and fresh medium is introduced to the cultures. After a 7-day expression period, cells are plated and mutation frequencies are determined based upon a Poisson distribution.

2.2.2b. HGPRT, Lymphocytes--In Vivo. The Strauss-Albertini test was originally developed as a direct technique for assessing the in vivo incidence of mutant cells arising in circulating lymphocytes in animals and man[40,41]. In the test, rare TG' peripheral blood lymphocyte (PBL) variants that incorporate ^{3}H-TdR in response to PHA, despite the presence of 6-thioguanine, can be enumerated by light microscopy after autoradiography (see Figure 8). The 42-hr culture period is of insufficient length to allow all responding lymphocytes to divide such that in vitro TG' PBL frequencies correspond to in vivo frequencies. Conditions for testing humans are those shown; however, rat lymphocytes are cultured 60 hr prior to adding ^{3}H-TdR. The test includes a cryopreservation step to eliminate "phenocopies," PBLs that are artifically TG' owing to the resistance capacity of cells already cycling at the moment of challenge.

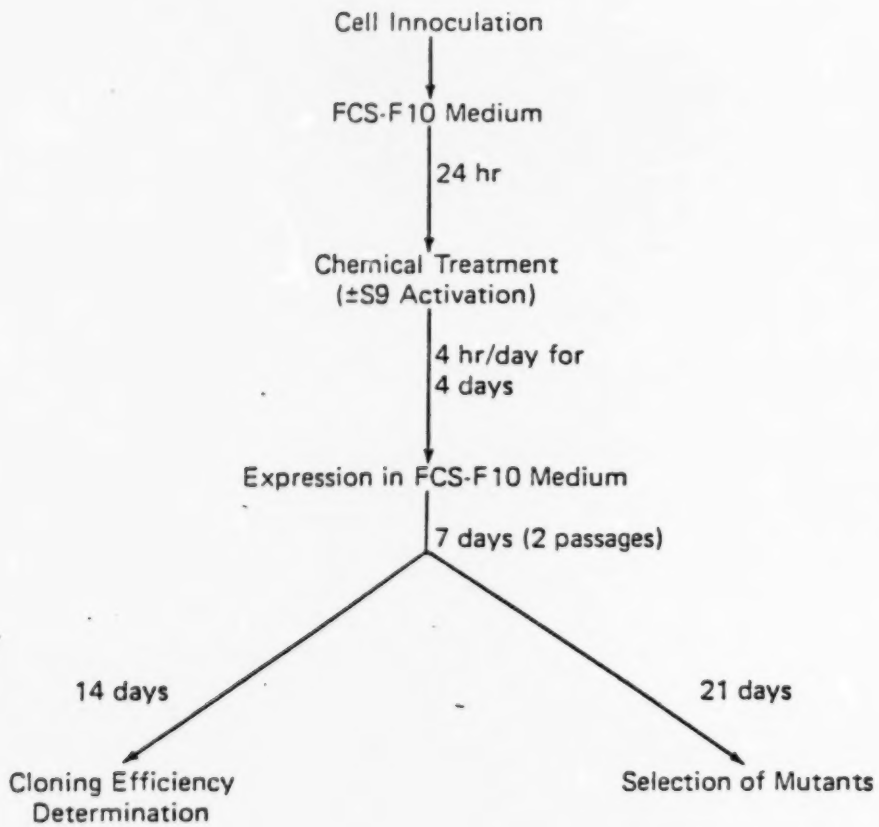


Figure 7. Induction of 6-thioguanine resistance studied in nonsynchronized human fibroblasts in the presence or absence of S9 activation.

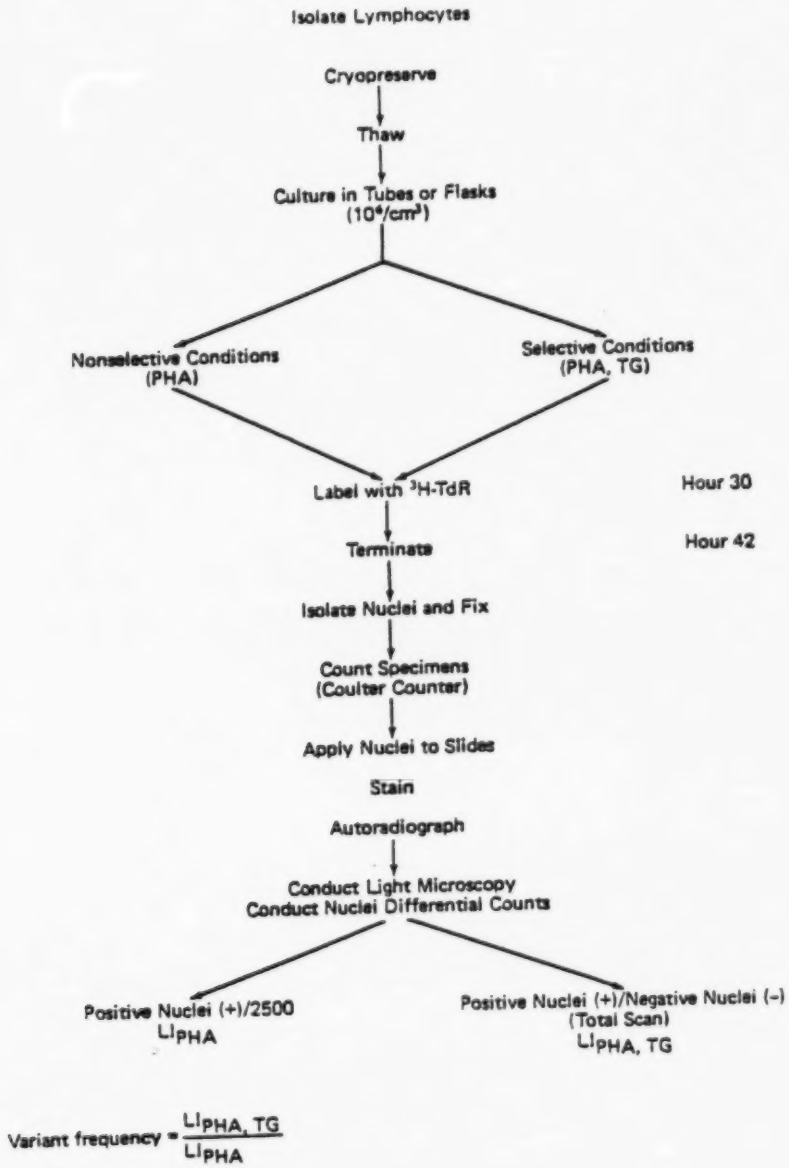


Figure 8. The Strauss-Albertini test procedure.

Variants are assumed to be HGPRT$^-$ mutants by virtue of various characterizations and analogy with the naturally occurring LN mutation. The test has been suggested as a means to diagnose LN heterozygosity[42]. Thioguanine-resistant PBLs have been found at frequencies ranging from 4 to 6×10^{-6} in normal individuals and at significantly higher frequencies in humans and animals receiving iatrogenic and experimental mutagen exposures, respectively[40-41]. The Strauss-Albertini test is well suited to long-term repeated monitoring due to the brevity of both latency and patency periods influencing variant frequencies after exposures to mutagens. Confirmation of the mutant nature of variants is now possible using the new lymphocyte clonal assay as follows:

The lymphocyte clonal assay is an adaptation of an immunological technique for cloning nontransformed T-lymphocytes from blood and other tissues[43]. By using T-cell growth factor, continuously growing T-lymphocytes (CTL) can be grown for months with maintenance of normal functions. Rare drug-resistant precursor cells can be isolated and expanded to form visible colonies in appropriate selective media containing T-cell growth factor. As depicted in Figure 9, lymphocytes are diluted in growth factor medium containing 0.05% X-irradiated sheep red blood cells, which serve as feeders; cells are then plated in paired 96-well microtiter plates at 10^5 cells per well in the presence of TG (final concentration = 2×10^{-5} moles) and at 1 cell/well in the absence of TG. Spent media are replaced with fresh media (at 5 and 9 days and labeling ^{3}H-TdR occurs on day 9). On day 10, activity per well is assessed by colorimetry, light microscopy, and scintillation spectrophotometry. Cloning efficiencies (CE) are calculated for each plate, assuming a Poisson

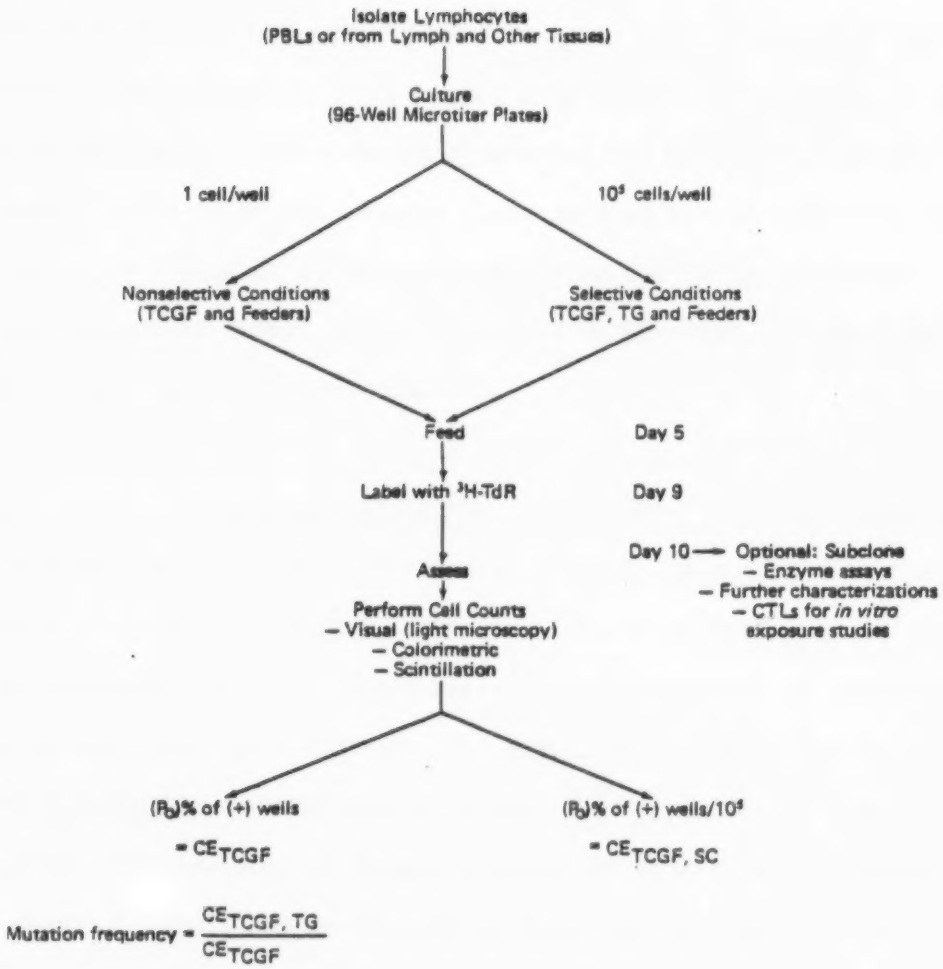


Figure 9. Lymphocyte clonal assay procedure.

distribution, such that the ratio $CE_{TCGF,TG}/CE_{TCGF}$ approximates *in vivo* or *in vitro* (for CTLs) mutation frequencies. The high sensitivity of this method can be confirmed by reconstruction experiments using mixtures of minority LN PBLs (and in further experiments, TG' CTLs cloned from normal individuals) in a majority of normal cells. The observed recoveries are satisfactory for detecting mutant cells in normal persons i.e., persons not at risk from mutagen exposures (mutation frequency is approximately 4 to 6 x 10^{-6}). Strauss-Albertini and lymphocyte clonal assays conducted in parallel yielded comparable results for frozen/thawed PBLs and CTLs[43].

2.2.2c. HGPRT, Lymphocytes--In Vitro. The lymphocyte clonal assay may be adapted to the evaluation of genotoxic damage after *in vitro* exposures to various agents (Figure 10). Individual susceptibility to mutagens and the relevancy of lymphocyte specific locus assays for mutation may be questioned in terms of detectable short-term effects. In addition, the assay may be used for mutagenicity testing in mice, because mice were used in the first immunological cloning studies. The response of target lymphocytes from diverse tissues to a variety of chemical agents may be assayed in mice (and in humans, to a lesser extent). Such studies would help to develop the means to extrapolate the results from experiments with rodents to humans.

2.2.2d. TK, Lymphoma Cells--In Vitro. The method for determining gene mutations at the thymidine kinase (TK) locus involves the use of a special isolated heterozygous cell line of L5178Y mouse lymphoma cells[44-46]. In this assay, $TK^{+/-}$-3.7.2C cells (6 x 10^6 cells/dose) are treated

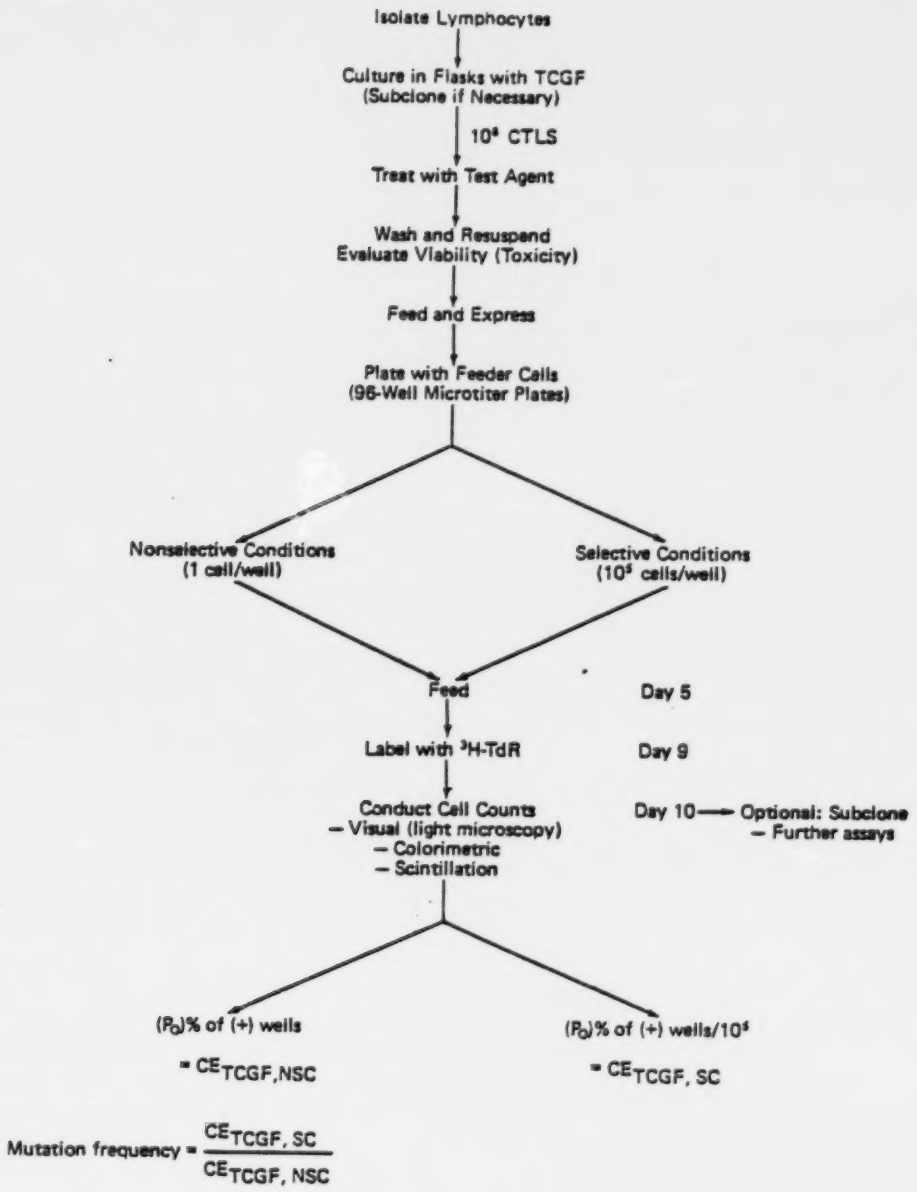


Figure 10. A possible procedure for the adaptation of the clonal assay to the evaluation of genotoxic damage after in vitro exposures to an agent.

with either a pure chemical test agent or a complex mixture for 4 hr. An Aroclor-induced rat liver S9 system can also be incorporated if exogenous activation is required. Following the 4-hr treatment, cells are washed free of test agent, resuspended in fresh growth medium, and the newly induced mutant cells allowed to express for 2 days. During expression, the cells are maintained in log-phase growth by performing cell counts and daily adjusting cell density to 0.2×10^6 cells/ml. Thymidine-kinase-deficient mutants are quantitated by cloning 3×10^6 cells/dose in 1 μg/ml trifluorothymidine, a thymidine analog toxic to TK-competent cells. Cells are incubated for 9 to 11 days to allow the formation of colonies that can be quantitated on an automatic colony counter. Because treated cells are also cloned without selective agent (600 cells/dose), the mutant number can be adjusted for plating efficiency and the data expressed as a mutant frequency (see Figure 11). Survival is calculated according to the method of Clive and Spector[47] and includes both a measure of growth in suspension and growth in cloning medium.

Recent research indicates that the detectable mutants include chromosomal aberrations affecting the expression of the TK locus as well as single gene mutations[48-50]. Because the type of mutant may correlate with the size of the mutant colony (see Figure 11), it may be possible to quickly determine relative clastogenic as well as mutagenic potential[50].

2.2.3 Methods for Measuring Exposure

Body fluid analysis using microbial mutagenesis tests provides a biological indication of exposure to mutagenic or promutagenic substances.

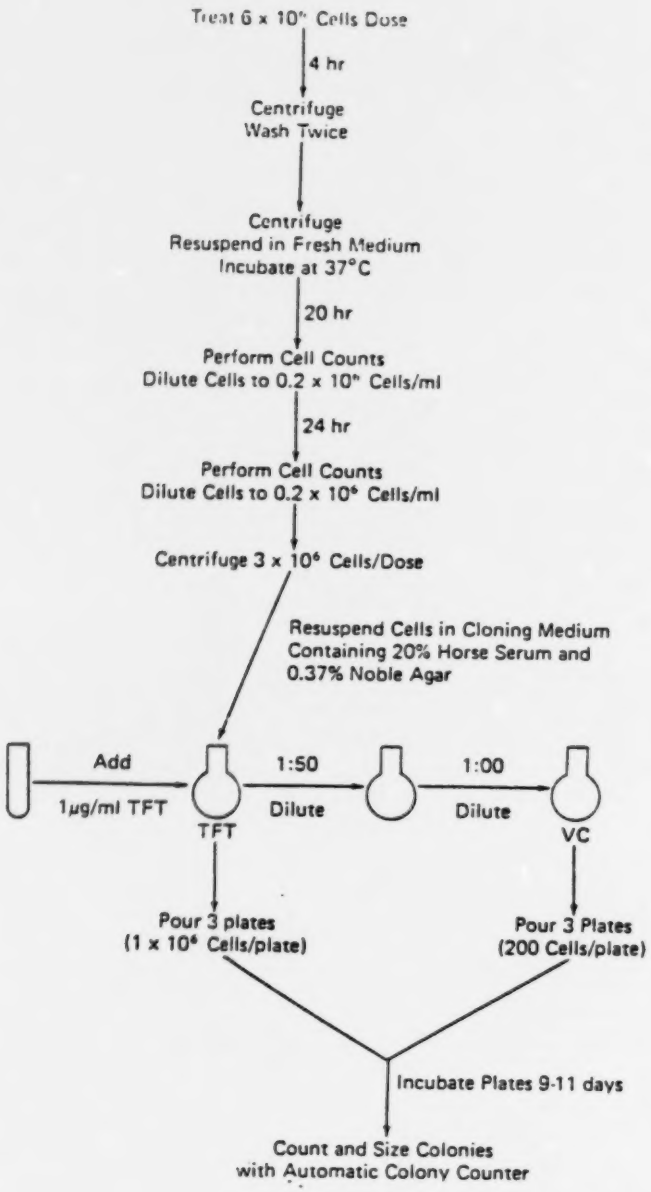


Figure 11. Protocol for the analysis of the thymidine kinase locus of L5178Y mouse lymphoma cells.

2.2.3a Urine Screening with Salmonella typhimurium. A major difficulty in environmental epidemiology is the measurement or estimation of exposure of individuals. Under certain conditions, urine screening allows investigators to determine whether individuals have been exposed to mutagens or promutagens and, in some circumstances, to make a semiquantitative estimate of individual exposure. The methodology used to screen urine for mutagens with bacterial systems was published first by Yamasaki and Ames[51]. Legator et al.[52] recently reviewed this method and other methods for screening various tissues and fluids for the presence of mutagens. The reader should refer to this review for a basic understanding of the techniques commonly used.

Urine monitoring (Figure 12) involves four major procedures: collection, storage, preparation, and bioassay of the urine sample. Care must be taken because each step is critical to the final result. It should be noted that urine monitoring provides only an estimate of exposure, and is not a measurement of a toxicological effect upon any individual or population.

In sample collection, each subject volunteer is given a thorough explanation of the study and is required to complete a questionnaire related to the study. The questionnaire, completed during an interview, is used to obtain information on potential exposure to agents other than those under investigation and indicates the volunteer's health status and attitude toward the study. When possible, the questionnaire is quantitative.

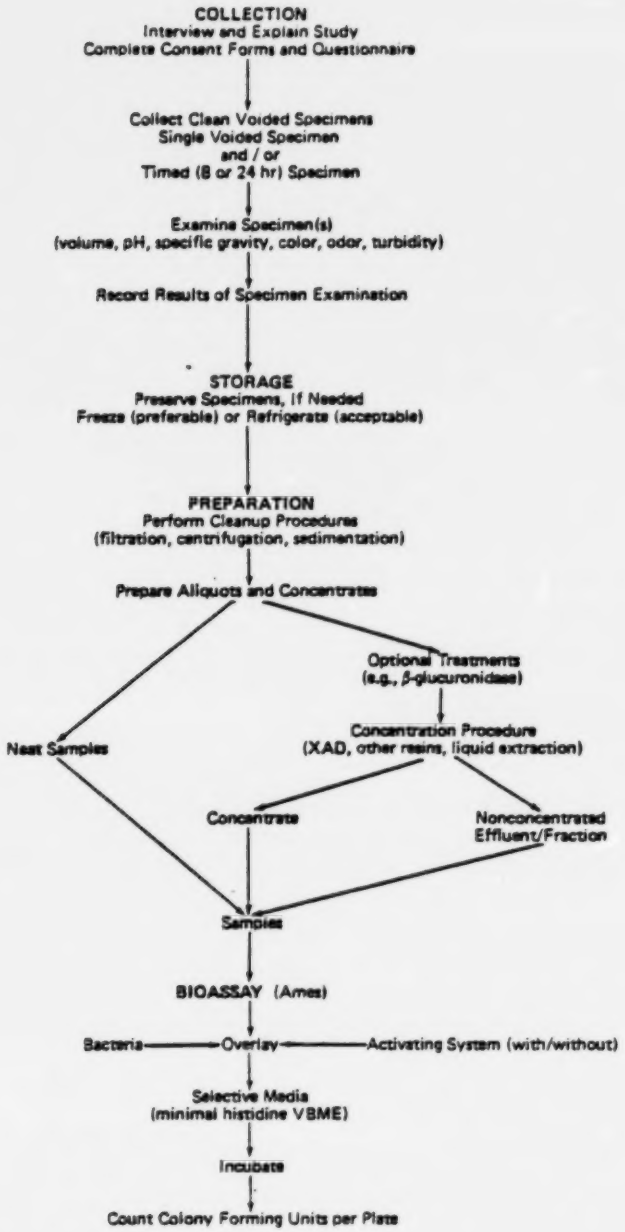


Figure 12. Urine monitoring with microbial mutagenicity screening.

After the interview, clean voided urine samples are collected. Two types of samples are collected--the occasional or single specimen and the timed specimen. The single sample is usually collected either at the first voiding after an exposure period or immediately after arising in the morning. The advantage of the morning specimen is that urine voided at this time is more concentrated and in a better state of preservation. Timed specimens are needed when attempting to quantitate the excretion rate of any substance. The more typical time period is 24 hr. The type of sample (single or timed) collected depends upon the type of exposure evaluated. After collection, each sample is examined for volume, pH, specific gravity, odor, color, etc.

Because samples frequently cannot be evaluated immediately, proper storage of the urine samples is essential. For most studies, samples are frozen immediately (e.g., on dry ice); however, simple refrigeration is adequate. Chemical preservatives are avoided because they may extract or otherwise interact with organic mutagens present.

Preparation of the sample for bioassay includes any "clean up" procedures and the aliquoting and/or concentrating of organics within each sample. Because urine samples contain bacteria, fibrous material, and cellular debris, they are filtered, centrifuged, or sedimented. In addition, because the concentration of mutagens may be low and because mutagens may also be bound to other biochemical species, samples are usually treated with a deconjugating enzyme and concentrated. The XAD2 resin technique is acceptable for concentrating mutagens from some types of agents (e.g., cigarette smoke) but less desirable for others,

especially chemotherapeutics. The original (neat) sample is evaluated as a check on mutagen concentration and the concentration method.

The bioassay method presently employed is the Ames Salmonella typhimurium plate incorporation assay. The urine sample is plated in the same manner as any liquid test sample. It should be recognized that some urine samples can contain enough histidine to give increased numbers of spontaneous revertants per plate and that false positive results are possible. Histidine may be removed from neat or concentrated samples by treating the samples with histidase. Data are analyzed and interpreted as described by Stead et al.[53].

2.2.4 Dosimetry Methods

The following methods are applicable to animals dosed with radiolabeled chemical and to other cell types after the initial cell separation step.

Lymphocytes are separated from blood serum and lysed in a buffer containing Tris Cl, pH 8, EDTA, and 1% SDS. The concentration of radiolabeled chemical is determined in whole blood, lymphocyte fraction, and lysate. The lysate is incubated 18 to 24 hr with 1 mg/ml pronase to digest protein. The lysate is then treated with an equal volume of phenol saturated with 50 mM Tris Cl and 1 MM EDTA. The aqueous phase containing the nucleic acid is extracted two additional times with phenol; RNA and DNA are then precipitated with alcohol. The nucleic acid is resuspended and treated with ribonuclease to obtain DNA. The purity of the fraction can be improved and ribonuclease removed by a subsequent treatment with pronase, phenol, and alcohol.

Purified DNA is degraded by acid hydrolysis[54,55] or by enzymatic degradation[56]. The DNA constituents--bases, nucleosides, and nucleotides--may be analyzed for total and specific DNA adducts.

3. RESULTS AND DISCUSSION

3.1 Comparative Evaluation of a Chemotherapeutic Agent--Cyclophosphamide

The data in Table I summarize preliminary evaluations of cyclophosphamide performed in this laboratory. The results are positive in the Salmonella TA100 strain in the presence of an S9 metabolic activation system. Positive results in this strain usually are indicative of base-pair substitution mutations. The compound is positive following multiple treatments in human fibroblasts at the HGPRT locus. Similarly, the agent is positive in _in vivo_ rat lymphocytes at the HGPRT locus[41] and for SCE induction in rodent cells _in vivo_[28] and _in vitro_ (with activation).

As described in Table I, positive effects were generated at a dose equivalent to 4 to 5 logarithmic dose units (LDUs), the most sensitive of these tests being gene mutation in the human fibroblasts.

The results of urine screening and direct testing of cyclophosphamide are shown in Table II. When _in vitro_ data from direct compound testing using the plate incorporation assay are compared with data obtained in screening the urine of a patient treated with cyclophosphamide, it may be seen that the exposure is detected and that metabolism of the compound has occurred to yield a direct-

TABLE I. EFFECTS OF CYCLOPHOSPHAMIDE IN SHORT-TERM TESTS

	Ames/Salmonella TA98	Ames/Salmonella TA100	Human BFU[a] (in vivo) TA100	Human Fibroblasts HGPRT (in vitro)	Rat Lymphocytes HGPRT
Point/Gene Mutation					
Qualitative results	-/-[b]	-/+	+/+	-/+	+/0
Quantitative results[c]				1 μg/ml	3 mg/kg
-log (10^{-5} x dose)[d]	---	4.1[e]	---	5	4.5
SCE Induction			Maternal Bone Marrow (in vivo)	Embryo (in vivo)	V79 Chinese Hamster Cell (in vitro)
Qualitative results			+	+	-/+
Quantitative results[c]			5 mg/kg	5 mg/kg	5 μg/ml
-log (10^{-5} x dose)[d]			4.3	4.3	4.3

[a]BFU = body fluid (urine test).
[b]Test results are shown without/with S9 activation.
[c]Approximate minimum dose required.
[d]Measure of sensitivity, 100,000 to 1 ppm equals 0 to +5 log dose units.
[e]Calculations based on a total plate volume of 32 ml.

TABLE II. COMPARISON OF URINE FROM TREATED PATIENTS WITH RESULTS OF TESTING OF TREATMENT COMPOUND

Sample	Activation[a]	Strain	Result
Urine:			
Cyclophosphamide Treatment	+	TA100	+
	-	TA100	+
	+	TA98	Neg
	-	TA98	Neg
Direct Testing:			
Cyclophosphamide			
	+	TA100	+
	-	TA100	Neg
	+	TA98	Neg
	-	TA98	Neg

[a]S9 activation used (+); S9 activation not used (-).

acting metabolite. It is thought that phosphoramide mustard represents one of the more potent cytotoxic metabolites of this compound[57].

The data for gene mutation and SCE can be compared with experimental data on chromosomal effects observed in humans. Adequate dosimetry information on cyclophosphamide exposure to patients is available, as shown in Table III. Since the LDU values in the table were calculated assuming the cumulative dose necessary for producing chromosomal damage, and since the activated metabolites of cyclophosphamide are excreted[58], the lower LDU values better reflect the lowest effective drug concentration. On a logarithmic scale, the dose required for effects in humans is similar to that used in animal and in vitro bioassays (Table I).

Although the data summarized in Table III were obtained from patients undergoing chemotherapy with cyclophosphamide, information was not available on subsequent tumor induction. The International Agency for Research on Cancer (IARC) has reported that more than 100 cases of cancer exist for patients receiving cyclophosphamide, although many patients are concomitantly given other cytostatic drugs and/or radiation[59]. In 1975, IARC reported that at least 10 cases of malignant tumors developed in patients treated with cyclophosphamide where there was no previous clinical evidence of cancer[60]. In view of the large number of people treated with cyclophosphamide, the data base for risk estimation is very small. Although the agent is considered a human carcinogen[59], its effectiveness in prolonging life in a variety of disorders is well established.

TABLE III. HUMAN DATA FOR CYCLOPHOSPHAMIDE

Chromosomal effects	Total Dose (g)	Logarithmic Dose Units -Log (10^{-5} mg/kg)[a]
SCE, In Vivo		
100 mg/day E = 22 days[b] Raposa[61]	2.2	3.5
Chromosome Aberration, In Vivo		
E = variable Schmid and Bauchinger[62]	1.5 to 1.7	3.7
250 mg/day E = 20 days Arrighi et al.[63]	5	3.1
E = 6 to 8 weeks Schmid and Bauchinger[64]	1.2 to 18.2	3.8
E = variable Bauchinger and Schmid[65]	1.5 to 18.8	3.7
3 to 5 mg/day E = 6 to 8 months Dobos et al.[66]	0.6	4.1

[a]Assuming a 70-kg human and 100,000 to 1 ppm equals 0 to +5 logarithmic dose units; the calculations are based on the lowest cumulative dose.
[b]E = duration of exposure.

The significance of the bioassay data with respect to possible cancer induction in humans can be investigated by examining the data of Wall and Clausen[67] on patients receiving very large doses of cyclophosphamide. Fatal urinary bladder carcinomas developed in four of the five patients treated with high doses. The patients received from 114 to 295 g of cyclophosphamide over a period of several years. These doses are generally 100-fold higher than those reported in Table III for chromosomal effects.

Thus, for cyclophosphamide, the bioassays detect effects occurring at similar doses (mg/kg) as do tests of chromosomal damage occurring in humans. However, these doses may be 100-fold less than the cumulative doses required for an 80% tumor incidence in humans. Whether the frequency of cancer induction is significant at these lower doses is unknown.

3.2 A Problem Carcinogen--Ethyl Carbamate

EC has been consistently negative for *in vitro* SCE induction and/or gene mutation in various mammalian cell systems[23,68]. However, it has tested clearly positive for *in vivo* SCE induction in several rodent species[69]. These discrepant findings illustrate the difficulties that may be encountered in testing common environmental chemicals under the parallelogram approach. Exogenous activation systems currently employed cannot always simulate *in vivo* metabolic processing. Thus, testing with *in vitro* methods exclusively can lead to misleading results.

Two related carcinogens, ethyl N-hydroxycarbamate (ENHC) and vinyl carbamate (VC), are believed to be metabolically derived from EC and, either directly or indirectly, to account for the carcinogenic activity associated with the parent compound. Early interest centered on ENHC, and potent teratogenic and genotoxic activities were revealed for this substance[70]. However, ENHC has been shown to possess considerably less carcinogenic potential than EC[70]. On the other hand, VC is a chemical of much current interest, and the few studies to date support its theoretical implication in EC carcinogenesis. Dahl et al.[71,72] have shown that VC is considerably more potent than EC for the induction of EC-type tumors in rats and mice and for gene mutations in Salmonella. They suggested that VC may be a metabolic intermediate of EC, with VC epoxide possibly representing the ultimate carcinogen. Specific adducts expected from this line of reasoning have been found[73].

Studies have shown that VC is far more reactive than EC or ENHC for inducing SCEs and gene mutations in a variety of in vivo and/or in vitro mammalian cell systems[74]. For example, Figure 13 illustrates SCE induction levels in mouse bone marrow cells after in vivo exposures to these carbamate chemicals. In this particular study, VC caused effects that were several times greater than EC or ENHC (up to 8 times the baseline SCE level) at less than one-tenth the dose of EC or ENHC. Negative results with 2-hydroxy ethyl carbamate (2-HEC), a noncarcinogen, were also seen. Tables IV and V reveal the relative in vitro activities of these carbamate chemicals in human lymphocytes and in V79 cells. Again, VC is clearly the most reactive; EC is essentially negative with or without S9 activation. In the V79 cell system, VC was direct-acting for the production of SCEs, although gene mutations were not

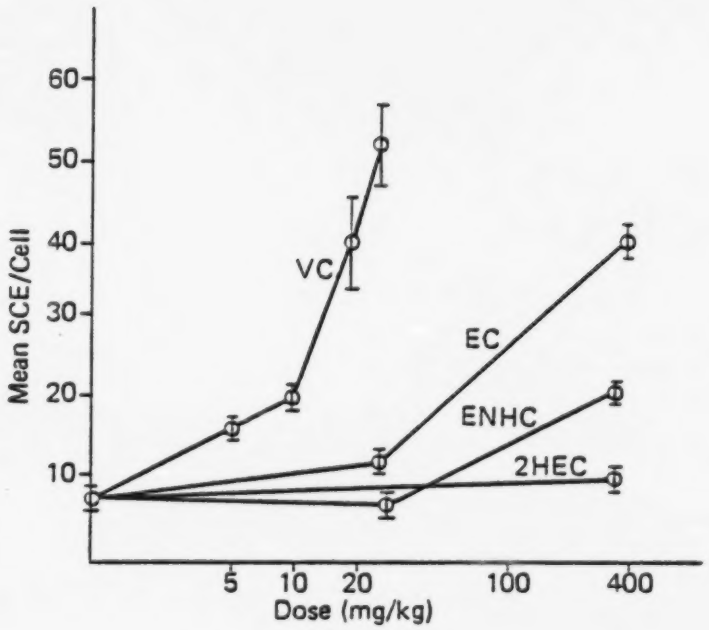


Figure 13. SCE induction in mouse bone marrow cells after <u>in vitro</u> exposure to vinyl carbamate, ethyl carbamate, ethyl N-hydroxycarbamate, and 2-hydroxyethyl carbamate. Each point represents the mean SCE frequency (± the standard error) usually calculated from 20 to 40 cells/animal, 3 or more animals/treatment. From Allen et all.(23)

TABLE IV. TOXICITY, GENE MUTATION, AND SCE INDUCED BY ETHYL CARBAMATE, VINYL CARBAMATE, ETHYL N-HYDROXYCARBAMATE, AND 2-HYDROXYETHYL CARBAMATE IN CHINESE HAMSTER V79 CELLS[a]

Chemical	Dose (ng/ml)	Toxicity (%)		Mutagenesis (6-TG) Mutants/10^6 cells		Mean SCE/cell[c]	
		+S9[b]	-S9	+S9	-S9	+S9	-S9
Ethyl carbamate	20.0	81	64	<10	<10	13 ± 4.4	9 ± 2.5
	10.0	81	77	<10	<10	12 ± 3.5	9 ± 2.7
	5.0	100	84	<10	<10	11 ± 4.0	9 ± 2.6
Vinyl carbamate	1.0	0.4	54	Toxic	<10	Toxic	82 ± 12.4
	0.2	6	90	591	<10	Toxic	30 ± 8.4
	0.01	52	53	236	<10	67 ± 8.5	15 ± 5.0
	0.001	48	58	69	<10	25 ± 5.9	14 ± 4.6
Ethyl N-hydroxy-carbamate	7.5	29	11	<10	62	22 ± 9.0	Toxic
	5.0	45	22	<10	55	18 ± 7.5	39 ± 14.2
	1.0	56	48	<10	51	15 ± 3.6	15 ± 3.6
	0.2	52	50	<10	72	20 ± 8.4	18 ± 9.1
	0.01	69	66	<10	20	14 ± 4.6	14 ± 5.0
2-Hydroxyethyl carbamate	10	86	77	29	<10	13 ± 4.4	13 ± 3.8
	5	68	87	<10	<10	16 ± 5.0	11 ± 4.0
	1	59	61	31	<10	15 ± 4.7	11 ± 3.0
	0.1	123	105	16	<10	16 ± 4.9	12 ± 3.7
Control	0	100	97	<10	<10	13 ± 3.5	11 ± 2.9
DMBA	0.015	65	-	222	-	46 ± 12.2	-
MNNG	0.0001	-	48	-	82	-	52 ± 14.3

[a]From Allen et al.[133].
[b]Aroclor-induced Syrian hamster S9.
[c]Mean ± standard deviation of 30 to 40 cells/treatment.

TABLE V. IN VITRO SCE FREQUENCIES IN HUMAN LYMPHOCYTES TREATED WITH ETHYL CARBAMATE OR VINYL CARBAMATE[a]

Test Chemical	Dose (mg/ml)	Number of Cells	SCE/Cell Ranges	SCE/cell[b]
Control	-[c]	54	3-14	7.4 ± 2.7
Ethyl Carbamate	1.0	65	2-15	8.3 ± 3.0
	5.0	54	4-14	7.9 ± 2.7
	10.0	34	3-20	8.2 ± 4.2
Vinyl Carbamate	0.01	31	3-17	9.1 ± 3.8
	0.10	27	17-67	41.4 ± 12.4
	0.20	28	17-66	39.3 ± 12.5

[a]From Allen et al.[22].
[b]Mean value ± standard deviation/sample of cells analyzed.
[c]All control and treatment group cells were grown with 25 μmoles BrdU.

induced. Increases up to 8 times the baseline level of SCEs resulted. Yet, with the addition of Aroclor-induced hamster liver S9 enzymes, the doses required to cause this effect greatly decreased, and extensive gene mutagenesis for 6-TG resistance resulted as well. Perhaps the coincident SCE and gene mutation effects stem from VC epoxide lesions, which are different from those caused by VC itself. ENHC was less reactive, and 2-HEC was essentially negative.

It has also been observed that Aroclor-induced S9 enzymes obtained from a mouse strain (A) with a high susceptibility to EC carcinogenesis can metabolically convert VC to more reactive genotoxic products than Aroclor-induced S9 enzymes from a mouse strain (C57BL/6) characterized by a low susceptibility to EC carcinogenesis (Figure 14)[69]. Significantly higher levels of SCE induction and gene mutagenesis result in V79 cells when the former mouse strain S9 preparation is used. That strain-specific differences in S9 metabolic conversions of VC parallel strain-specific differences in carcinogenesis susceptibilities to EC lends some support to the proposition[71,72] that VC may be a critical intermediate in EC pathways to tumor formation. Additional experimentation is aimed at confirming the metabolic relationship between EC and VC and at further clarifying the role and fate of the latter substance.

VC has also been tested in the L5178Y/$TK^{+/-}$ mouse lymphoma assay. In this system, VC was found to give positive mutagenicity at the TK locus, both with and without an Aroclor-induced rat liver S9 (Figure 15). As with the V79 assay, the dose required to give a genotoxic effect was lower with S9 activation. An analysis of the size of the mutant colonies induced (Figure 16) indicates that

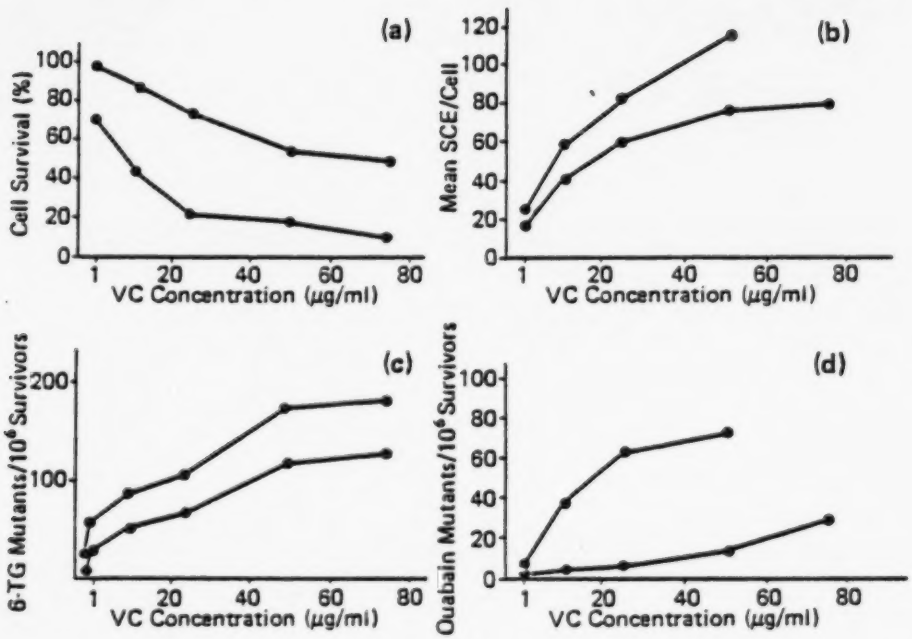
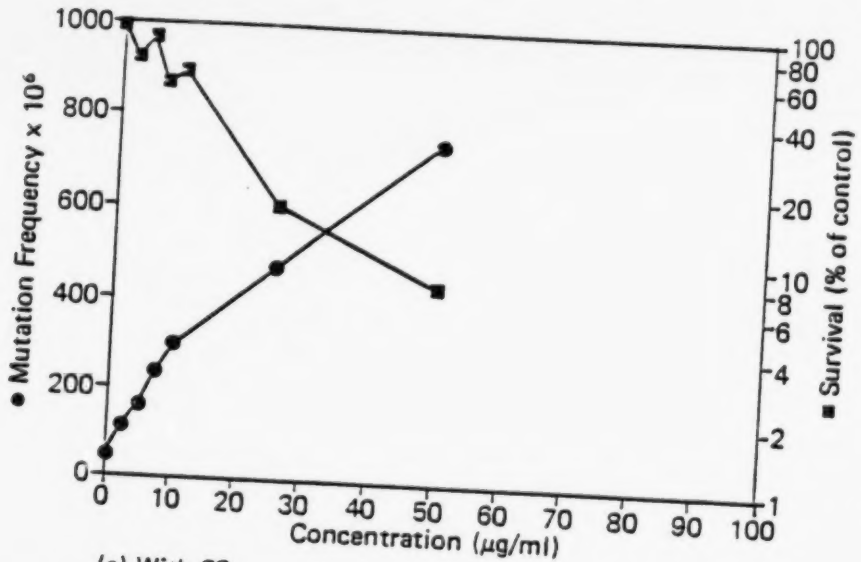
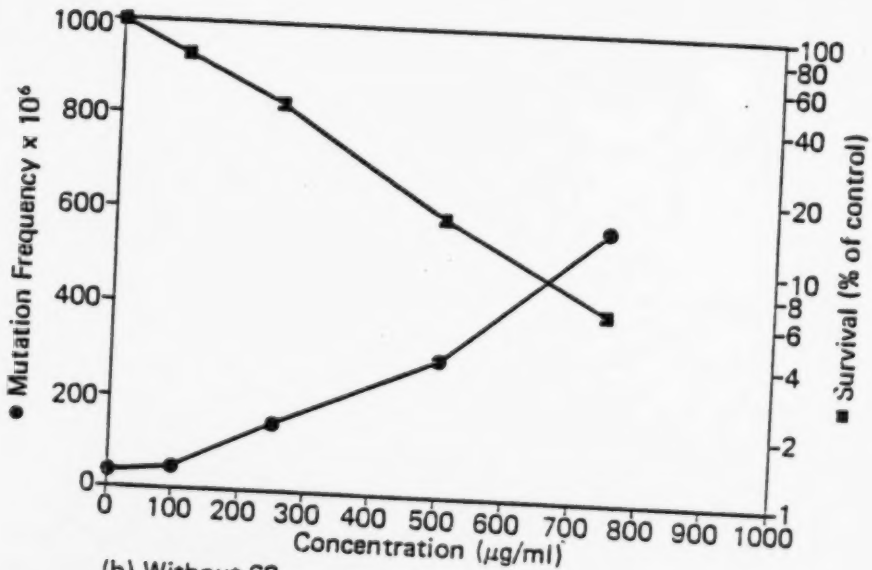


Figure 14. Comparison of capabilities of A vs. C57BL/6 mouse strain liver enzyme preparations (Aroclor-induced S9 fractions) to activate vinyl carbamate to induce various genotoxic effects in V79 cells. Cytotoxicity (a) is shown as percent cell survival of the control value (100%), SCE frequencies as mean per cell (b), gene mutation frequency for 6 thioguanine resistance (c), and gene mutation frequency for ouabain resistance (d) as number of mutants/10^6 survivors minus control value. These values represent an average of 3 experiments. From Sharief et al. (69)



(a) With S9.



(b) Without S9.

Figure 15. Mutagenicity of vinyl carbamate at the TK locus of L5178Y mouse lymphoma cells with S9 (a) and without S9 (b) activation.

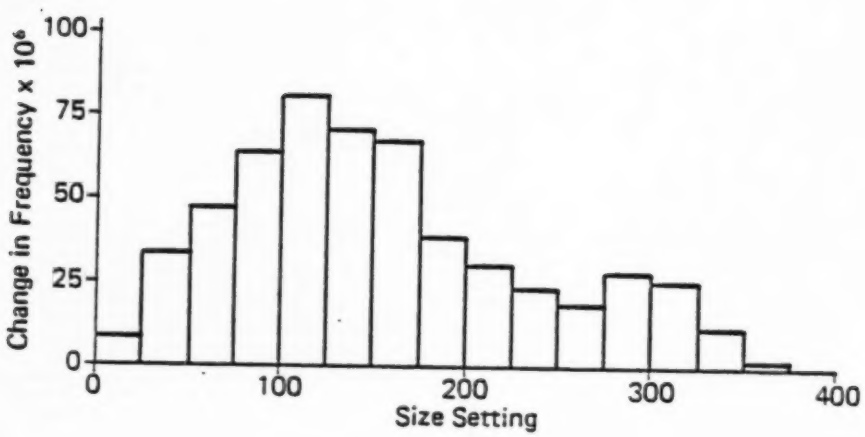


Figure 16. Colony sizing of trifluorothymidine-resistant mutants following treatment with 750 µg/ml (without S9 activation). Small colony peak is to the left; large colony peak is to the right.

VC does produce a significant proportion of small colony mutants, which have, as a class, been shown to represent chromosome aberrations affecting chromosome 11[48-50], which carries the TK gene[75]. VC has also been shown to be an efficient inducer of gross aberrations in these L5178Y/$TK^{+/-}$ mouse lymphoma cells[76].

3.3 Dosimetry

A common denominator in our methodology that has not yet been considered is the cellular or molecular (DNA) dose determined for the component test systems. Dose-response data are used to estimate the effects that might occur under similar exposure conditions in humans. These extrapolations are facilitated by using peripheral blood lymphocytes in a number of the component procedures. This cell type can be studied directly in in vitro cultures, and in in vivo systems where the cells are obtained from exposed animals or exposed humans. However, a number of complicating factors exist in basing extrapolation procedures on cellular or molecular dose.

After exposure in a human or an experimental animal, the concentration of an agent may change rapidly under physiological conditions. Many chemicals are rapidly metabolized and are removed from the blood plasma within minutes to hours; the concentration in other tissues and the concentration at the molecular targets is similarly time dependent. Perhaps more important with regard to the present investigation is the fact that various repair mechanisms are operative to remove the agent or its metabolites, once their targets have been reached. In addition, it is possible that the cellular or molecular damage sustained will

not be expressed as an altered phenotype. Thus, careful experiments must be planned to allow deductions to be made concerning the effects of cellular metabolism and repair processes on the relationship between dose and the phenotypic expression of genetic damage in various tissues and species.

More specifically, the feasibility of basing dosimetry efforts on DNA adducts formed from labeled material may be questionable since the modified target may be unstable and the label may be liberated to the intracellular environment, even though a stable base change in DNA or other modification in the molecular target may have been produced. These considerations may be important in understanding recent experiments[77] where comparisons of SCE induction and adduct formation revealed that no demonstrable DNA product was formed at the same ratio as SCE, and, in fact, differences were 7- to 25-fold. Other experiments have shown that substantial alkylation of messenger RNA does not alter specific gene products[78]. Covalent binding data are not readily translated into information regarding the amount of target modification that occurs or may be transcribed after replication of DNA. Finally, isotopically labeled material cannot be used to monitor human exposure.

A recent report by Topal and Baker[79] indicates that methylation of the DNA nucleotide pool may be an important consideration in assessing molecular dose. Methylation of the DNA nucleotide precursor pool was shown to be 190 to 13,000 times that of the equivalent residue in the DNA helix itself.

Nevertheless, determining cellular or total dose and adducts in cell cultures and in experimental animals is relatively straightforward, with several

limitations. The agent must be readily available and appropriately labeled isotopically. The specific radioactive activity of the compound and its active metabolite must be sufficient to permit DNA or adducts to be isolated and counted. Ideally, quantitation would be based on relatively stable adducts. For example, alkyl derivatives of oxygen atoms are stable for more than 10 days for DNA treated in vitro[5]. Alkyl phosphotriesters are very stable and the half-life of other alkyl derivatives may be several days. Quantitation of such relatively stable derivatives of DNA is essential in relating genotoxic effects to exposure doses in human populations.

In summary, the question of dosimetry is very complex, and further experimental efforts are required before conclusions can be reached about the appropriateness of various possible measurements or about the most useful ways to integrate these measurements into the overall evaluation of the parallelogram approach.

4.0 SUMMARY AND CONCLUSIONS

Current methods of risk assessment for chemicals found in the environment are based on estimates of human exposure and associated effects. Agents are defined as cancer-causing only if exposure and effects data exist from case reports or clinical findings. Otherwise, such agent definition depends upon descriptive epidemiological studies where cancer incidence has varied with exposure to the agent or where there are similar findings in case-control and cohort epidemiological studies[59]. Unfortunately, such data can be assembled only after inadvertent exposure of individuals or certain populations. Clearly,

other prospective procedures are necessary for dealing with potentially hazardous agents found in the environment.

Data from animal experiments or short-term tests do not have the limitations of case reports in epidemiology, although problems are encountered in extrapolating data from in vitro and in vivo test systems to the human situation. It should be possible, however, to construct a laboratory model that would closely approximate the case for human exposure and effects.

The purpose of this paper has been to describe preliminary experiments and methodology directed toward the development of such an experimental model. This model should be useful in integrating somatic- and germ-cell testing or monitoring data with information on exposure. The rationale for such a model is based upon the parallelogram method described previously. The component parts of the model that we have assembled consist of in vitro and in vivo methods for measuring point/gene mutation and chromosomal effects. Obviously, there are many other genetic endpoints and related effects that could contribute to the detection and quantitation of chemically induced genetic damage in humans. Our research program is at its inception and lacks a number of components before a complete integration of our efforts will be feasible. Proper dosimetry is needed, and these measurements must be standardized (e.g., against direct radiation exposures). Genetic test systems must be made as uniform as possible and internal controls must be added. Despite these difficulties, our preliminary results may be subjected to an integrated assessment that is interpretable within the overall parallelogram concept.

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FIGURES

1a. Parallelogram for extrapolation to gene mutation *in vivo*.

1b. Parallelogram for extrapolation to gene mutation in germinal cells in humans.

2. Expanded concept of parallelogram method considering external and internal exposure.

3. Protocol for culturing human lymphocytes for SCE analysis.

4. Protocol for *in vitro* analysis of chromosomal, gene, and cytotoxic effects from mutagens/carcinogens.

5. Protocol for *in vivo* analysis of SCE induction in rodent bone marrow cells.

6. Induction of 6-thioguanine resistance studied in synchronized human fibroblasts in the presence or absence of S9 activation.

7. Induction of 6-thioguanine resistance studied in nonsynchronized human fibroblasts in the presence or absence of S9 activation.

8. The Strauss-Albertini test procedure.

9. Lymphocyte clonal assay procedure.

10. A possible procedure for the adaptation of the clonal assay to the evaluation of genotoxic damage after *in vitro* exposures to an agent.

11. Protocol for the analysis of the thymidine kinase locus of L5178Y mouse lymphoma cells.

12. Urine monitoring with microbial mutagenicity screening.

13. SCE induction in mouse bone marrow cells after *in vivo* exposure to vinyl carbamate, ethyl carbamate, ethyl N-hydroxycarbamate, and 2-hydroxyethyl carbamate. Each point represents the mean SCE frequency (± the standard error) usually calculated from 20 to 40 cells/animal, 3 or more animals/treatment. From Allen et al.[23].

14. Comparison of capabilities of A vs. C57BL/6 mouse strain liver enzyme preparations (Aroclor-induced S9 fractions) to activate vinyl carbamate to induce various genotoxic effects in V79 cells. Cytotoxicity (a) is shown as percent cell survival of the control value (100%), SCE frequencies as mean per cell (b), gene mutation frequency for 6 thioguanine resistance (c), and gene mutation frequency for ouabain resistance (d) as number of mutants/10^6 survivors minus control value. These values represent an average of 3 experiments. From Sharief et al.[69].

15. Mutagenicity of vinyl carbamate at the TK locus of L5178Y mouse lymphoma cells with S9 (a) and without S9 (b) activation.

16. Colony sizing of trifluorothymidine-resistant mutants following treatment with 750 μg/ml of vinyl carbamate (without S9 activation). Small colony peak is to the left; large colony peak is to the right.